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The MADS-Domain Protein AGAMOUS-Like 15 Accumulates in Embryonic Tissues with Diverse Origins[1]

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AGL15 (AGAMOUS-like 15), a member of the MADS-domain family of regulatory factors, accumulates preferentially in the organs and tissues derived from double fertilization in flowering plants (i.e. the embryo, suspensor, and endosperm). The developmental role of AGL15 is still undefined. If it is involved in embryogenesis rather than some other aspect of seed biology, then AGL15 protein should accumulate whenever development proceeds in the embryonic mode, regardless of the origin of those embryos or their developmental context. To test this, we used AGL15-specific antibodies to analyze apomictic embryogenesis in dandelion (*Taraxacum officinale*), microspore embryogenesis in oilseed rape (*Brassica napus*), and somatic embryogenesis in alfalfa (*Medicago sativa*). In every case, AGL15 accumulated to relatively high levels in the nuclei of the embryos. AGL15 also accumulated in cotyledon-like organs produced by the *xtc2* (*extra cotyledon2*) mutant of Arabidopsis and during precocious germination in oilseed rape. Furthermore, the subcellular localization of AGL15 appeared to be developmentally regulated in all embryogenic situations. AGL15 was initially present in the cytoplasm of cells and became nuclear localized before or soon after embryogenic cell divisions began. These results support the hypothesis that AGL15 participates in the regulation of programs active during the early stages of embryo development.

In seed plants, the sporophyte generation initiates with a unique developmental phase: the seed or embryo phase. During this phase, morphogenetic programs operate to establish the correct embryo form, storage reserves accumulate, desiccation tolerance is acquired, and a quiescent state is established that can be exited in a controlled manner at a later point. The early stages of embryo development have been difficult to study because the cells constituting the embryo are small, few in number, and embedded in several layers of maternal tissues. Much progress has been made recently in isolating genes encoding regulatory factors expressed during early embryogenesis (Li and Thomas, 1998; Lotan et al., 1998; Luerssen et al., 1998; for reviews of other genes, see Mordhorst et al., 1997; Berleth, 1998); however, relatively little is known about the corresponding gene products in terms of activity and/or regulation.

Although it is not restricted to embryos, the MADS-box regulatory factor *AGL15* (*AGAMOUS-like 15*) is expressed at approximately 10-fold higher levels during the embryonic phase than during any other phase of the life cycle (Heck et al., 1995; Rounsley et al., 1995). Members of the MADS-domain family contain a highly conserved, 55- to 60-amino acid motif (the MADS domain) that functions as both a DNA-binding and dimerization domain (for review, see Riechmann and Meyerowitz, 1997). MADS-domain proteins are found in plants, animals, and fungi and often play critical roles in the control of development. In Arabidopsis and snapdragon, for example, proteins such as APETALA1/SQUAMOSA, AGAMOUS/PLENA, APETALA3 / DEFICIENS, and PISTILLATA / GLOBOSA play central roles in the specification of meristem and floral organ identity (for review, see Riechmann and Meyerowitz, 1997). MADS-box gene families are quite large in plants but not in animals and fungi. At least 15 different members may be present in ferns (Münster et al., 1997) and more than 28 have already been identified in Arabidopsis (Riechmann and Meyerowitz, 1997). *AGL15* is one of the most divergent members of the family in Arabidopsis (Rounsley et al., 1995) and is the only member isolated to date that is expressed preferentially during embryogenesis.

AGL15-specific antibodies and immunohistochemistry were used previously to demonstrate that AGL15 accumulation and localization are developmentally regulated during zygotic embryogenesis. AGL15 accumulates in the cytoplasm of cells of the female germ unit before fertilization and moves into the nuclei after the first few cell divisions in the embryo, suspensor, and endosperm (Perry et al., 1996). Relatively high levels of AGL15 are maintained in the embryo nuclei throughout the period of morphogenesis and then decline as the embryo matures. Based on this accumulation pattern and the fact that it is similar in dicot and monocot embryos, we have proposed that AGL15 plays a conserved regulatory role during the early stages of the embryonic phase (Heck et al., 1995; Perry et al., 1996). If this role is important for the process of embryogenesis, AGL15 should accumulate in the nuclei in all young em-

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Abbreviations: DAP, days after pollination; DAC, days after the start of culture.

bryos and new embryonic organs regardless of when and how they initiate. To test this idea, we used AGL15-specific antibodies to examine a variety of developmental situations in a variety of flowering plants in which embryos or embryonic organs arise outside of the seed context or by means other than the fertilization of an egg. In every case we found that whenever embryos or embryonic organs were present, relatively high levels of AGL15 could be detected in the nuclei.

MATERIALS AND METHODS

Plant Material

Oilseed rape (*Brassica napus* cv Tower) plants were grown in a controlled-environment chamber (Conviron, Pembina, ND), under a 16-h light (370 μE m^{-2} s^{-1} at flower level) and 8-h dark regime (at 15°C and 10°C, respectively). Flowers on the primary inflorescence were hand-pollinated and tagged on the day they opened. For protein gel-blot analyses, zygotic embryos were collected at 30 DAP. Pea (*Pisum sativum* cv Little Marvel) plants were grown under the same conditions and zygotic embryos were collected at the early-cotyledon stage.

Arabidopsis (Landsberg *erecta* ecotype) wild-type plants and plants homozygous for the *xtc2* mutation were grown at 22°C in constant light (88 μE m^{-2} s^{-1}). For collection of secondary cotyledons and leaves, seeds were sown on germination medium containing Murashige and Skoog salts and vitamins (Murashige and Skoog, 1962) supplemented with 10 g L^{-1} Suc, 0.5 g L^{-1} Mes, and 7 g L^{-1} agar, pH 5.6 to 5.7, chilled at 4°C for 2 d, and grown for 10 to 12 d. R.S. Poethig (University of Pennsylvania, Philadelphia) provided the *xtc2* mutant seed.

Dandelion (*Taraxacum officinale*) flowers of various stages from small buds to open flowers were collected on the University of Wisconsin-Madison campus during the spring of 1996.

Alfalfa (*Medicago sativa*) fruits were collected from field-grown plants during the summer of 1998. S. Austin-Phillips (Biotechnology Center, University of Wisconsin, Madison) provided mature leaf tissue for the production of somatic embryos. Clones of a specific alfalfa genotype derived by a cross between Regen S and Regen Y, Regen-SY-27 (Bingham, 1991), were grown in an environmental chamber under a 16-h light (300 μE m^{-2} s^{-1}) and 8-h dark regime (at 21°C and 19°C, respectively).

Maize (*Zea mays*) zygotic embryos (early-maturation stage) were recovered by dissecting kernels removed from sweet corn obtained at a local grocery store during July and August of 1998.

Precocious Germination of Oilseed Rape Embryos

Using tungsten knives, we excised the developing embryos aseptically from 47-DAP seeds and placed them into culture on Monnier's embryo medium (Monnier, 1976) containing 1% Suc (w/v) in glass jars as described by Fernandez (1997). The embryos were allowed to develop for 27 d at 25°C under constant cool-white fluorescent light.

Oilseed Rape Microspore Embryogenesis System

Microspore embryogenesis was performed as described by Crouch (1982). We collected the oilseed rape flower buds (2.7–3.8 mm in length) from the primary inflorescence before the first flower opened. At this stage anthers contained uninucleate microspores or early binucleate pollen. We rinsed the flower buds in sterile water and aseptically removed the anthers and placed them dehiscence-line-up on medium as described by Crouch (1982). The cultures were incubated in the dark at 30°C for 2 weeks, after which time they were maintained in the dark at room temperature. We prepared the embryos, unorganized masses, and mature pollen derived from anther culture for immunohistochemistry analysis as described previously by Perry et al. (1996).

Alfalfa Somatic Embryogenesis System

Somatic embryogenesis was performed as described by Austin et al. (1995) with some modifications. We collected the first and second fully expanded dark-green trifoliate leaves of alfalfa genotype Regen-SY-27 (Bingham, 1991; Austin et al., 1995). Leaf surfaces were sterilized by washing for 30 s in 70% (v/v) ethanol, followed by 90 s in 10% (v/v) household bleach and 0.1% (v/v) Tween 20, and then rinsed three times in sterile, distilled water. We cut off the leaf margins on moist filter paper and placed the remaining explants onto B5 medium (Brown and Atanassov, 1985) with 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin. After 3 to 4 weeks under constant light at room temperature, the explants had callused and many small green pro-embryos were present. At this point we moved the explants onto B5 medium without hormones to allow the pro-embryos to develop. After another 2 to 3 weeks under constant light at room temperature, the embryos had established bilateral symmetry and could be moved to Murashige and Skoog medium (Murashige and Skoog, 1962) to allow the roots and shoots to develop.

Antibody Preparation

Heck et al. (1995) previously described the production of polyclonal antibodies that recognize AGL15. To ensure specificity of the antiserum for AGL15, the highly conserved MADS domain was removed and the remaining domains of oilseed rape AGL15–1 were overexpressed in *Escherichia coli* to produce antigen. For protein gel-blot analysis, antibodies that recognized the bacterial antigen were blot-affinity purified from antiserum as described by Tang (1993). The serum that remained after the antigen-coated strips were removed was "immunodepleted" and we used it as the control in several experiments. For immunohistochemistry, immune and preimmune sera were preadsorbed against mature oilseed rape leaf pieces to remove serum components that bind nonspecifically to fixed plant tissues, as described by Perry et al. (1996).

Immunodetection on Protein Gel Blots

Tissues were flash-frozen in liquid N_2 and soluble protein extracts were prepared as described previously (Heck et al., 1995). Proteins in the extracts were separated on denaturing 15% (w/v) polyacrylamide gels before blotting onto Immobilon PVDF membranes (Millipore). Blots were incubated with affinity-purified antibodies (diluted 1:1000 or 1:1500 [v/v] relative to the original immune serum), preimmune serum, or immunodepleted serum. The immunoreactive protein was visualized using the Lumi-GLO system (Kirkegaard and Perry Laboratories, Gaithersburg, MD), with the secondary antibody diluted 1:5000 (v/v). We exposed the blots to x-ray film (Biomax MR, Kodak) for 1 to 3 min.

Immunohistochemistry

Localization of AGL15 on tissue sections (7 μm) in paraffin embedding medium (Paraplast Plus, Sigma) was performed as described previously (Perry et al., 1996). To prepare figures, we scanned the slides with a film scanner (RFS 2035, Kodak) and assembled the images onto plates using PhotoShop 3.0 (Adobe Systems, Mountain View, CA).

RESULTS

Demonstration of Antibody Specificity

We prepared antibodies that were highly specific for AGL15 for use in protein gel-blot analyses and localization experiments. Only the portions of the oilseed rape AGL15–1 gene product downstream of the MADS domain (i.e. the portions that distinguish AGL15 from other members of the MADS-domain family) were used to elicit the immune serum. Antibodies that recognize these portions of AGL15 were subsequently isolated from the immune serum by affinity purification. When the affinity-purified antibodies were used in protein gel-blot analyses, a single major immunoreactive protein with an apparent molecular mass close to that predicted by the nucleotide sequence (30 kD) appeared in the soluble protein extracts prepared from oilseed rape embryos (Fig. 1a). Preimmune serum did not recognize this protein, and immune serum that had been immunodepleted by incubating it with excess antigen exhibited a significant reduction in reactivity (Fig. 1a).

We showed previously that the epitopes recognized by antibodies in the immune serum were associated with the nuclei in the developing embryos of oilseed rape, Arabidopsis, and maize (Perry et al., 1996). When preimmune serum (Perry et al., 1996) or immunodepleted serum (not shown) was used in immunolabeling experiments, no staining was visible. The immune antiserum did not label nuclei in wild-type Arabidopsis flowers (Fig. 1b) or wild-type oilseed rape inflorescence apices (Perry et al., 1996), despite the relative abundance of other MADS-domain proteins in these tissues. This result demonstrates specificity for this family member and also eliminates the possibility that the antibodies recognized proteins that reside permanently in the nucleus. When sections of flowers from transgenic Arabidopsis plants that constitutively overexpressed AGL15 (G.R. Heck, S.E. Perry, S.E. Patterson, and D.E. Fernandez, unpublished data) were labeled, we saw intense, nuclear-associated immunostaining in all cells (Fig. 1c).

The affinity-purified AGL15 antibodies also recognized a related set of epitopes in other plant species. One major immunoreactive band was present in the soluble protein extracts prepared from the zygotic embryos of a variety of flowering plants (Fig. 1d). In many dicots the immunoreactive protein migrates with a molecular mass similar to that seen in oilseed rape (Heck et al., 1995). In other dicots and in monocots, however, the immunoreactive protein

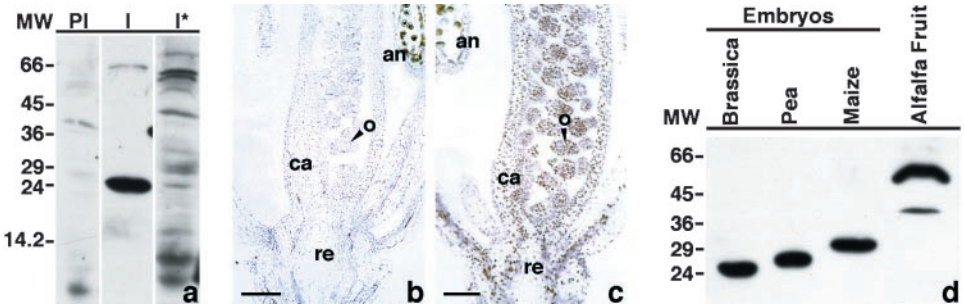

Figure 1. Specificity of AGL15 immune serum. a, Protein gel blot of soluble protein extracts prepared from developing oilseed rape embryos. Each lane contained 150 μg of protein. Identical strips were incubated with either preimmune serum (PI), affinity-purified antibodies (I), or immunodepleted immune serum (I*). All serum preparations were at the same dilution (1:1000) relative to the original sera. b, Central whorls of a late-stage, pre-anthesis flower from a wild-type Arabidopsis plant treated with AGL15 immune serum. Immunostaining can be detected in association with pollen nuclei at this stage, but is not associated with other nuclei in floral tissues. c, Localization of AGL15 in the central whorls of a late-stage, pre-anthesis flower from a transgenic Arabidopsis plant that is constitutively expressing AGL15. Immunostaining can be detected in association with the nuclei in virtually all cells in the flower. d, Protein gel blot of soluble protein extracts prepared from zygotic embryos (oilseed rape, pea, and maize) or fruits with developing embryos (alfalfa) probed with affinity-purified antibodies that recognize AGL15. Each lane contained 150 μg of protein. an, Anther; ca, carpel; o, ovule; re, receptacle. Bars = 75 μm.

has reduced mobility and larger apparent molecular mass (Fig. 1d). The molecular basis of the mobility shift seen in pea (approximately 2 kD) and maize (approximately 5 kD) embryos is not yet known. In developing alfalfa fruits, we saw two major immunoreactive bands. The larger of these corresponded to the single immunoreactive band in alfalfa zygotic and somatic embryos (Fig. 4f) and was approximately twice the size of the immunoreactive protein in pea. The other immunoreactive protein, which had an apparent molecular mass of approximately 40 kD, was less abundant and may have been associated with maternally derived tissue.

We demonstrated previously that the immunoreactive protein in maize showed the same expression pattern, relative abundance, and cellular localization during zygotic embryogenesis as AGL15 in oilseed rape and Arabidopsis. Based on these data, we concluded that the immunoreactive protein in monocots (despite its altered size) is likely to represent the functional equivalent, if not the true homolog, of AGL15. All of the evidence we have accumulated to date, including the results presented in this paper, is consistent with the conclusions that (a) the immune antiserum recognizes a restricted set of epitopes and (b) the majority of these epitopes are associated with low-abundance proteins whose attributes (e.g. accumulation pattern and cellular localization) suggest a structural and/or functional relationship to AGL15.

AGL15-Related Proteins Accumulate in Embryos That Arise by Apomixis

In plant species that reproduce by apomixis, embryo development is not tied to a fertilization event. In diplospory, a type of gametophytic apomixis, the megaspore mother cell does not undergo meiosis normally and there is no reduction in chromosome number (for review, see Koltunow, 1993; Calzada et al., 1996). Mitotic divisions give rise to an embryo sac in which the cells are still diploid. A common apomict of this type is dandelion, in which mitosis of an unreduced but otherwise normal egg gives rise to an embryo that is genetically identical to the mother plant.

Sections of developing dandelion seeds were labeled with AGL15 antiserum. As shown in Figure 2a, immunoreactive protein was present in cells constituting the embryo sac before embryo development initiated and in the surrounding ovule tissue. Immunoreactive protein accumulated in the cytoplasm and was excluded from the nuclei of cells in the embryo sac at this stage of development (Fig. 2a). At the eight-cell stage, we could detect immunoreactive protein in both the cytoplasm and nuclei of cells in the embryo (Fig. 2b). At the late-globular stage, immunoreactive protein was predominantly associated with the nuclei of cells and was present in all cell layers and tissue types of the embryo (Fig. 2c). This pattern of accumulation is identical to that previously described for AGL15 in sexually derived embryos (Perry et al., 1996), and was not observed when preimmune serum was used (data not shown).

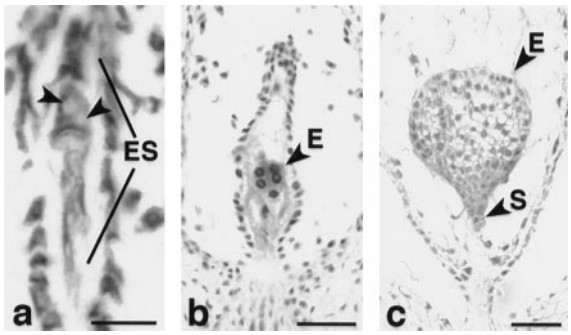

Figure 2. Accumulation of AGL15 during apomixis in dandelion. Tissue sections treated with anti-AGL15 antiserum and visualized by bright-field microscopy are shown. a, Dandelion embryo sac (ES). Immunoreactive protein is present in the cytoplasm of cells in the embryo sac. At this stage of development, immunoreactive protein is excluded from embryo sac nuclei (indicated with arrowheads). Labeling with anti-AGL15 antiserum is also apparent in the surrounding tissues of the seed. Bar = 20 μm. b, Dandelion seed at the eight-cell stage of embryo development. Immunoreactive protein is present in the cytoplasm and nuclei of cells of the preglobular-stage embryo (E). Labeling with anti-AGL15 antiserum is also apparent in other tissues of the seed. c, Dandelion seed at the late-globular stage of embryo development. Immunoreactive protein is associated with nuclei in the embryo (E) and suspensor (S). Lower levels of labeling are present in other seed tissues. Bars = 50 μm.

AGL15 Accumulates in Supernumerary Cotyledons Produced at the Shoot Apex

We previously showed that Arabidopsis *lec1–2* embryos, which prematurely exit the embryonic phase and produce cotyledons with leaf-like traits (Meinke et al., 1994; West et al., 1994), showed a premature loss of AGL15 around the transition stage of development (Perry et al., 1996). Another interesting set of Arabidopsis mutants with regard to changes in organ identity are the *extra cotyledons* (*xtc*) mutants such as *xtc2*, which produce seedlings with one to two supernumerary, cotyledon-like organs (Conway and Poethig, 1997). To determine whether these organs contained elevated levels of AGL15, lateral organs that appeared after germination were harvested from 10- to 12-d-old *xtc2* seedlings. Because *xtc2* seedlings develop more slowly than wild-type Landsberg *erecta* seedlings, organs isolated from *xtc2* seedlings were not at the same developmental stage as leaves isolated from wild-type seedlings of the same age. To obtain samples that were developmentally equivalent, we took advantage of the incomplete penetrance of the *xtc2* mutant.

Approximately 30% of the seedlings produced organs that resembled cotyledons. Organs of this type, which have at most one trichome, were collected as "secondary cotyledons." Another 30% of the seedlings produced only normal-looking leaves, and organs were collected from these seedlings for the leaf sample. The remaining 40% of the seedlings produced organs with a small number of trichomes. We discarded them because we could not reliably assign them to either group (cotyledon or leaf). We probed the blots of soluble protein extracts prepared from the collected organs with anti-AGL15 affinity-purified an-

tibodies. As shown in Figure 3a, organs displaying embryonic identity (secondary cotyledons) accumulated higher levels of AGL15 than organs of the same age displaying "nonembryo" or "leaf" identity. We could not detect AGL15 in the soluble protein extracts prepared from the leaves or primary cotyledons of 10-d-old wild-type Landsberg *erecta* seedlings (data not shown).

Precociously germinating oilseed rape embryos also produce new lateral organs that take on a cotyledon identity (Finkelstein and Crouch, 1984; Fernandez, 1997). As shown in Figure 3, b and c, when immature oilseed rape embryos were excised from late-maturation-stage seed (44–47 DAP) and placed into culture, three different types of organs were produced at the shoot apex. Typically, the first primordium developed into an extra cotyledon and the second primordium into a leaf (Fig. 3b), but primordia occasionally gave rise to chimeric organs with large sectors of cotyledon and leaf tissue (Fig. 3c). We collected the organs from precociously germinating embryos 27 DAC and sorted them into different types (secondary cotyledon, leaf, and chimeric organ).

We probed the blots of soluble protein extracts prepared from these three samples with affinity-purified anti-AGL15 antibodies. AGL15 accumulated to relatively high levels in the secondary cotyledons (Fig. 3d). An additional, more diffuse immunoreactive band showing reduced mobility (equivalent to a molecular mass difference of approximately 7 kD) was also visible in this sample. AGL15 accumulated at lower levels in the chimeric organs (Fig. 3d). The leaves did not accumulate detectable amounts of AGL15 (Fig. 3d). Therefore, the amount of AGL15 in each sample varied according to the amount of embryonic tissue in that organ type.

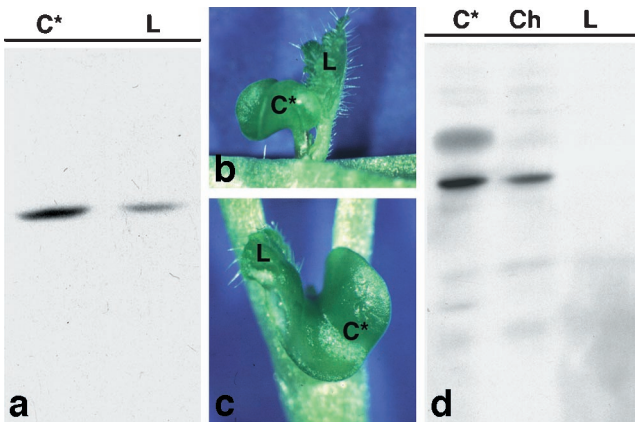

Figure 3. Accumulation of AGL15 in embryonic organs produced at the shoot apex. a, Protein gel blot of soluble protein extracts prepared from secondary cotyledons (C*) or leaves (L) of 10- to 12-d-old *xtc2* Arabidopsis seedlings probed with affinity-purified antibodies that recognize AGL15. Each lane contained 150 μg of protein. AGL15 accumulated to higher levels in the secondary cotyledon sample than in the leaf sample. b, Organs produced at the shoot apex of an oilseed rape embryo excised from the seed at 44 DAP, after several weeks in culture. The first primordium developed into a secondary cotyledon (C*), and the second primordium developed into a leaf (L). The embryonic cotyledons are out of the frame to the right. c, Chimeric organ produced at the shoot apex of an oilseed rape embryo excised at 44 DAP, after several weeks in culture. Large sectors of cotyledon (C*) and leaf (L) tissue are visible. d, Protein gel blot of soluble protein extracts prepared from secondary cotyledons (C*), chimeric organs (Ch), or leaves (L) produced by oilseed rape embryos excised at 47 DAP, cultured for 27 d, and probed with affinity-purified antibodies that recognize AGL15. Each lane contained 150 μg of soluble protein. The level of accumulation of AGL15 is approximately proportional to the amount of cotyledon tissue present in the sample.

AGL15-Related Proteins Accumulate in Embryos That Arise from Somatic Tissue

Plants have the unique ability to produce embryos from somatic cells (for review, see Zimmerman, 1993). Somatic embryogenesis occurs in many species and from both diploid and haploid progenitor cells. One well-developed system in which embryos arise from cells that have undergone meiosis involves culture of excised anthers or isolated microspores (for review, see Touraev et al., 1997). Oilseed-rape microspores will develop into haploid embryos instead of pollen grains if environmental stress is applied, usually in the form of heat shock (Crouch, 1982; Touraev et al., 1997). As shown in Figure 4a, microspores developing inside an excised anther accumulated low levels of AGL15. When whole anthers were used for microspore embryogenesis, embryos were produced at a low frequency, along with pollen and unorganized masses. One such unorganized structure obtained by anther culture appears in Figure 4b. When sections of this unorganized structure were labeled with AGL15 antiserum, we observed no nuclear staining (Fig. 4c). Other structures had a distinct epidermal layer, reflecting development in a more organized, embryogenic mode. One such structure, shown in Figure 4, d and e, showed strong nuclear labeling with AGL15 antiserum. Mature pollen derived from either normal development or development in culture did not show labeling with the AGL15 antiserum (data not shown).

In some cases mature vegetative organs can also give rise to embryos. In alfalfa, somatic embryos are produced from explants taken from mature, fully expanded trifoliate leaves (Austin et al., 1995). When these explants are placed onto a culture medium with hormones, they develop into loosely adhering calli (unorganized masses) with small, green pro-embryos within 3 to 4 weeks. The embryos will mature if they are removed to a medium without hormones, and they can eventually grow into mature plants.

We examined the status of AGL15-related proteins in samples collected at various times after the start of culture in the alfalfa somatic embryogenesis system. As shown in Figure 4f, an immunoreactive protein band of approximately 40 kD was present in the excised leaf material. The level of accumulation (on a per-milligram-of-protein basis) was comparable to that seen for AGL15 protein in oilseed rape embryos: the protein accumulated at the upper end of the scale for AGL15 but was still a low-abundance protein. The 40-kD protein was the predominant immunoreactive protein during the first 2 d in culture. By 3 DAC, the levels of the 40-kD protein had started to decline, and an additional immunoreactive protein with an apparent molecular

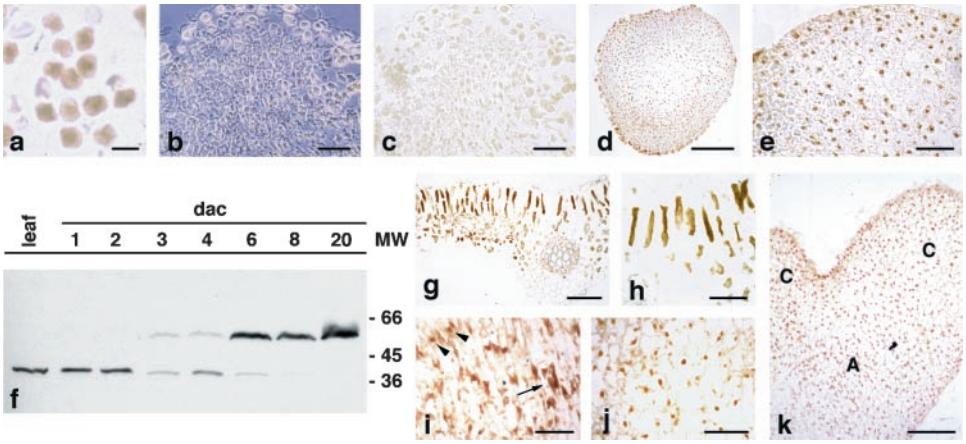

Figure 4. Accumulation of AGL15 during somatic embryogenesis. a, Oilseed rape developing microspores in an anther of the appropriate stage for embryo culture probed with anti-AGL15 antiserum. AGL15 is present at low levels in the cytoplasm and nuclei of developing microspores. Bar = 10 μm. b, Unorganized mass produced during oilseed rape anther culture viewed with phase-contrast optics. No organized epidermal layer is apparent. Bar = 50 μm. c, Unorganized mass shown in b viewed with bright-field optics. No nuclear labeling is observed with the AGL15 antiserum. Bar = 50 μm. d, Oilseed rape microspore embryo derived from anther culture probed with anti-AGL15 antiserum. An organized epidermal layer is present, indicating development in an embryonic mode. AGL15 is present at relatively high levels in the nuclei of cells throughout the embryo. Bar = 200 μm. e, Higher-magnification view of the embryo shown in d to show nuclear subcellular localization of AGL15. Bar = 50 μm. f, Protein gel blot of soluble protein extracts from alfalfa leaf explants in a somatic embryo culture system probed with affinity-purified antibodies that recognize AGL15. Each lane contained 125 μg of protein. The major immunoreactive protein band in extracts prepared from mature leaf tissue migrated at approximately 40 kD. Within several DAC, another immunoreactive band appeared that migrated at approximately 56 kD. The lower molecular mass gradually disappeared with increasing time in culture. The higher-molecular-mass band appeared gradually and persisted throughout the period of somatic embryogenesis. g, Tissue section of an alfalfa mature leaf probed with anti-AGL15 antiserum. The immunoreactive protein was predominantly associated with the mesophyll cells. Bar = 100 μm. h, Higher-magnification view of the mesophyll cells in the tissue section shown in g. The immunoreactive protein was associated with the cytoplasm. Bar = 50 μm. i, Tissue section of an alfalfa leaf explant 4 DAC probed with anti-AGL15 antiserum. Immunoreactive protein was predominantly associated with the cytoplasm in some cells (arrow) and with nuclei in other cells (arrowheads). Bar = 50 μm. j, Tissue section of an alfalfa leaf explant 7 DAC probed with anti-AGL15 antiserum. The immunoreactive protein was associated exclusively with the nuclei. Bar = 50 μm. k, Tissue section of a somatic embryo derived from alfalfa leaf culture probed with anti-AGL15 antiserum. The immunoreactive protein was associated with nuclei throughout the embryo, including the cotyledons (C) and the axis (A). Bar = 100 μm.

mass of 56-to-60 kD could be detected (Fig. 4f). By 6 DAC the 56- to 60-kD form had become the predominant form, and by 8 DAC, when the leaf pieces had started to callus, the immunoreactive protein band at 40 kD had largely disappeared (Fig. 4f). The 56- to 60-kD protein was the predominant immunoreactive species for the rest of the culture period (Fig. 4f) and was the only form that could be detected in zygotic and somatic embryos (not shown). When we examined seedlings regenerated from somatic embryos, approximately equal quantities of both forms of immunoreactive protein were present (not shown).

Using immunohistochemistry, we examined the subcellular localization of the immunoreactive protein(s) in this system. In the mesophyll cells in mature leaf tissues, it was present predominantly in the cytoplasm (Fig. 4, g and h). At 4 DAC immunoreactive protein was predominantly nuclear in portions of the explant but remained cytoplasmic in other cells (Fig. 4i). By 7 DAC, however, immunoreactive protein was associated with the nuclei in all of the cells in the explant (Fig. 4j). At subsequent times (data not shown) and in somatic embryos (Fig. 4k), the immunoreactive protein was exclusively nuclear. Therefore, the change in forms from 40 to between 56 and 60 kD occurred during the same period as the change in subcellular localization from cytoplasmic to nuclear, and it preceded the appearance of embryos in this culture system.

DISCUSSION

Embryonic Tissues Accumulate AGL15 or AGL15-Related Proteins

We reported previously that AGL15 accumulated at its highest levels in the products of double fertilization (the embryo, suspensor, and endosperm) following sexual reproduction in angiosperms and was maintained at high levels in immature embryos (Perry et al., 1996). If AGL15 is important for development in an embryonic mode, as this pattern of accumulation might suggest, we would also expect to find relatively high levels of AGL15 or AGL15-related proteins in developmental contexts where embryos or organs with embryonic tissue arise outside of the seed or by means other than fertilization of the egg. We have used AGL15-specific antibodies to examine developmental

situations of this type in a variety of flowering plants. In every case, whenever embryos or embryonic organs were present, relatively high levels of AGL15-related proteins could be detected as well.

The results of our study of dandelion embryos indicate that the absence of a fertilization event does not preclude accumulation of AGL15. In dandelion, embryos arise via apomixis, which is a form of asexual reproduction. AGL15-related protein accumulates in the same pattern in these asexual embryos as it does in zygotic embryos that arise following a fertilization event (Perry et al., 1996).

The results of our study of the *xtc2* mutant of Arabidopsis indicate that AGL15 accumulates in organs with embryonic features (i.e. cotyledons) even if they initiate during later stages of embryogeny and develop largely after germination. In the *xtc2* mutant of Arabidopsis, secondary cotyledons appear as a result of changes in the relative timing of embryo and shoot apex development (Conway and Poethig, 1997). *xtc2* embryo development is delayed, but shoot apex development initiates precociously. Maturation-stage *xtc2* embryos are at the late-heart to early-torpedo stage of development in terms of morphogenesis but have large primordia at the shoot apex (Conway and Poethig, 1997). We found that AGL15 accumulated to higher levels in the secondary cotyledons that developed from these primordia than in leaves of the same age.

The results of our study of organs produced during precocious germination in oilseed rape indicate that AGL15 also accumulates in cotyledons that are initiated outside of the seed environment. When immature oilseed rape embryos were excised and placed into culture, they produced three different kinds of organs: secondary cotyledons, leaves, and chimeric organs, with large sectors of cotyledon and leaf tissue (Finkelstein and Crouch, 1984; Fernandez, 1997). The amount of AGL15 present in these organs was directly proportional to the amount of cotyledon tissue present, despite the fact that these organs initiated after the maternal tissues were removed and often after an extended period in culture.

Finally, the results of our studies of somatic embryogenesis indicate that AGL15 accumulates even when embryos arise de novo from cells in other phases of the life cycle. Immature oilseed rape microspores can be induced to develop into pollen, calli, or embryos. AGL15 was undetectable in the nuclei of fully mature pollen and in the cells in unorganized masses. Only the cells that took on an embryonic identity accumulated higher levels of AGL15. The accumulation pattern of AGL15-related proteins was even more interesting in alfalfa, where embryos were obtained by culturing mature leaf tissue. Immunoreactive protein was present at relatively high levels in the leaf mesophyll cells of alfalfa.

Detectable amounts of AGL15-related proteins did not accumulate in the mature leaf tissues of oilseed rape, Arabidopsis (Heck et al., 1995; Perry et al., 1996; and S.E. Perry, personal observation), tomato (M.D. Lehti, personal observation), or maize (S.-C. Fang, personal observation); however, they did accumulate in the mature leaf tissues of pea, another legume (M.D. Lehti, personal observation). Does the accumulation of AGL15-related protein at high levels somehow enhance the embryogenic potential of alfalfa leaf cells? AGL15 accumulation did not appear to be sufficient to confer an embryonic identity on a cell or cells: the floral organs of plants overexpressing AGL15 accumulated AGL15 (Fig. 1c) but maintained their identity as reproductive tissues. However, AGL15 may be necessary to either direct or support development in an embryonic mode. The fact that immature oilseed rape microspores accumulated low but detectable amounts of AGL15 and also had embryogenic potential that can be expressed in culture may be significant in this regard.

Developmental Regulation of Localization of AGL15

In every situation in which embryogenesis was newly initiated, we have seen a change in the localization of AGL15 or AGL15-related protein. We demonstrated previously that localization of AGL15 was developmentally regulated in zygotic embryos of oilseed rape (Perry et al., 1996) and found that a similar change in subcellular localization, from cytoplasmically associated to nuclear associated, occurred at the start of embryogenesis in the asexual embryos of dandelion. Therefore, the change in epitope localization does not appear to be tied in any obligatory way to a fertilization event. The maternal environment also appears to be dispensable. In the alfalfa somatic embryo culture system, immunoreactive protein was initially present in the cytoplasm of leaf mesophyll cells and became exclusively localized in the nuclei even before somatic embryo development was apparent. These results indicate that the change in the localization of AGL15-related proteins is not dependent on information from maternal tissues of the seed.

The importance of subcellular localization as a mechanism for regulation of transcription factors is increasingly being recognized (for review, see Calkhoven and Ab, 1996; Jans and Hübner, 1996; Vandromme et al., 1996). In addition to AGL15, there are other plant MADS-domain proteins that show differential localization. In snapdragon, DEFH125 was present in the cytoplasm in the vegetative cell of pollen grains but was present in the nuclei of cells in the transmitting tissue of the upper portion of the style after pollination (Zachgo et al., 1997). Regulation of localization may be tied to heterodimer formation in the case of the Arabidopsis MADS-domain proteins AP3 (APETALA3) and PI (PISTILLATA). In plants that ectopically express AP3 protein fused to the reporter GUS, GUS activity was confined to the cytoplasm unless the appropriate partner (PI in this case) was also present (McGonigle et al., 1996). Similarly, fusions between PI and GUS were cytoplasmically localized unless AP3 was present (McGonigle et al., 1996). Unlike AP3-PI, AGL15 could bind DNA as a homodimer in vitro (Perry et al., 1996). Therefore, the formation of a heterodimer between AGL15 and another MADS-domain protein may not be a prerequisite for nuclear import.

In the alfalfa somatic embryo system, the developmentally regulated change in subcellular localization of AGL15-related epitopes coincided with a change in the apparent

molecular mass from 40 kD to between 56 and 60 kD. Although it is possible that the major immunoreactive protein bands represent proteins that are unrelated to AGL15, we consider this unlikely, based on the high degree of specificity that the antibodies displayed and the correspondence between the abundance, expression pattern, and localization of the immunoreactive proteins in alfalfa and those of AGL15 in other species. Alfalfa had two very distinct forms of AGL15-related proteins that were both present during the period when vegetative tissue (leaf) was making a transition to embryogenesis. It is intriguing that precociously germinating oilseed rape embryos, which were also in a state of developmental transition, contained two forms of immunoreactive protein as well (Fig. 3d).

In alfalfa, the shift in molecular mass was coincident with and may reflect a regulatory event that was tied to the change in subcellular localization. This shift could involve degradation of the cytoplasmic form and synthesis of a new nuclear form, possibly via alternate splicing to generate a new transcript. Alternatively, the cytoplasmic form may be posttranslationally modified in some way. Possible posttranslational modifications include dimerization, phosphorylation, glycosylation, and/or attachment of ubiquitin-like proteins (Calkhoven and Ab, 1996; Jans and Hübner, 1996; Vandromme et al., 1996; Haltiwanger et al., 1997; Johnson and Hochstrasser, 1997). We will be investigating these and other possibilities in future studies to determine the basis of the change in molecular mass in both alfalfa and oilseed rape and its relationship to subcellular localization.

In summary, we have shown that AGL15 or AGL15-related proteins accumulated at higher levels in the nuclei whenever a set of cells developed in an embryonic mode, regardless of their origin or the particular developmental situation. The fact that nuclear-associated forms of immunoreactive protein appeared at early stages as a result of a developmentally regulated process supports the idea that these proteins play an important role at the beginning of embryogenesis. Future experiments will be aimed at more thoroughly defining that role and the contribution that AGL15 and AGL15-related proteins make to the establishment of the new sporophytic generation.

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